Volume 4 Number 2 February 1977 Nucleic Acids Research

DNA replication in Physarum polycephalum: characterization of DNA replication products made in vivo in the presence of cycloheximide in strains sensitive and resistant to cycloheximide

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Received 7 December 1976

ABSTRACT

Synchronous plasmodia of cycloheximide-sensitive and cycloheximide-resistant strains of Physarum polycephalum were labelled with ^{3}[H]-deoxyadenosine in pulse and pulse-chase experiments in presence and absence of cycloheximide. The replication products were studied with alkaline sucrose gradient sedimentation analysis. We show that the action of cycloheximide on DNA replication in Physarum is mediated through the ribosome, since the ribosomally located resistance also makes the plasmodial DNA replication refractile to the action of cycloheximide. Cycloheximide caused inhibition of three stages in DNA replication in the wild type: first, the formation of primary replication units ("Okazaki" size fragments), secondly, the ligation of primary units into secondary ("Replicon" size) units and thirdly, the ligation of secondary units into mature DNA.

INTRODUCTION

In a previous communication we showed that DNA replication in Physarum polycephalum proceeds with formation of primary and secondary intermediate replication products, with mature DNA resulting only after a long period of ligation (Funderud and Haugli, 1975)[1]. This resolution of molecular intermediates in DNA replication allowed the study of details of the effects of cycloheximide on DNA synthesis in Physarum reported here. This analysis is of particular value since we have a characterized strain of Physarum polycephalum resistant to cycloheximide. Cycloheximide inhibits protein synthesis in eukaryotes by binding to a protein of the large subunit of the ribosome, thereby preventing the elongation step in polypeptide synthesis. (Rao and Grollman, 1967[2], Jimenez, Littlewood and Davies, 1971[3]). Cycloheximide also inhibits DNA synthesis in eukaryotes (Kerridge, 1958)[4]. This effect has usually been taken to reflect a need for

continued protein synthesis in order for DNA replication to proceed (Cummins and Rusch, 1966[5], Gautschi and Kern, 1973[6]). In the present work, a cycloheximide resistant strain whose resistance is known to be ribosomal (Haugli, Doye and Jimenez, 1972)[7] is used to get firm evidence for the mode of action of cycloheximide on DNA replication. Earlier results on the effects of cycloheximide on DNA synthesis in Physarum include studies by Cummins and Rusch (1966)[5], Muldoon et al. (1971)[8] and Werry and Wanka (1972)[9]. Most workers assume that the effect of cycloheximide on DNA synthesis is caused by the lack of specific protein(s) that can not be provided under cycloheximide inhibition. It is uncertain whether initiation of new replication units, propagation of the replication fork or both are sensitive targets. Weintraub and Holtzer (1972)[10] using embryonic chick erythroblasts and either cycloheximide or puromycin found no evidence for inhibition of initiation of new DNA chains, but could account for the 50 % drop in DNA synthesis entirely by a reduced rate of chain growth. Similar results were reported by Gautschi and Kern (1973)[6] and Gautschi (1974)[11]. In these experiments one should take "new DNA chains" to mean autoradiographically resolvable "replication units" in the meaning of Huberman and Riggs (1968)[12]. With higher resolution of the molecular mechanisms involved, werner and Maier (1975)[13] concluded that the joining of primary replication units ("Okazaki" pieces) was extremely sensitive to the inhibition of protein synthesis. At the same time, direct chain elongation was going on at a reduced rate.

The present work is a continuation of a previous report on the intermediates of DNA replication in Physarum polycephalum (Funderud and Haugli, 1975)[1]. Here, we investigate the fate of these replication intermediates in a cycloheximide sensitive and a cycloheximide resistant strain in the presence and absence of the drug.

MATERIALS AND METHODS

Strains and culture techniques: The wild type, cycloheximide sensitive, strain used here is TU291 (Funderud and Haugli, 1975)[1]. The cycloheximide resistant strain carried the mutation actA 169 which was isolated by Haugli (1971)[14] and shown to be a

ribosomal mutation conferring cycloheximide resistant protein synthesis on the plasmodium (Haugli, Doye and Jimenez, 1972)[7]. The medium was a modified semidefined medium (Daniel and Baldwin, 1964)[15]. Culture conditions have been described (Funderud and Haugli, 1975)[1].

Labelling procedures and alkaline sucrose gradient analysis.

Methods are similar to those described before (Funderud and Haugli, 1975)[1]. Here, we have pretreated and chased 0,65 cm^2 cultures (containing $4x10^6$ nuclei or about 2 µg of DNA) on 15 ml reservoirs of medium containing cycloheximide at 5 µg/ml or deoxyadenosine at 1,3 mM. Pulses were done by placing the culture, plasmodial side down, on a 40 µl droplet of the radioactive medium. This contained ^{3}[H]-thymidine (Amersham Code TRK·418), or ^{3}[H]-deoxyadenosine, (New England Nuclear Code NEN·123) at radioactive concentrations 500 or 250 µCi/ml. After nuclear isolation and alkaline lysis (20°C) gradients were centrifuged for 4,5 hours in SW40 rotor of the Spinco L65B centrifuge at 40 000 rpm, 20°C, for analysis of pulse and pulse-short chase experiments. Conditions were 3 hours at 30 000 rpm for analysis of pulse-long chase experiments.

Marker DNA

Phage λ DNA marker for the alkaline sucrose gradients were prepared as before. S Values and molecular weights were calculated as previously described (Funderud and Haugli, 1975)[1].

The corrected S value of the primary, Okazaki size, replication unit was calculated with the aid of ^{32}P-polydeoxythymidine of chain length 160 nucleotides, kindly provided by Dr. K. Kleppe, University of Bergen, Norway.

RESULTS

1. Precursor effects.

It was shown by Bersier and Braun (1974)[16] and Evans et al. (1976)[17] that cycloheximide causes an expansion of the pool of dTTP (and dCTP) in Physarum, leaving the pool of dATP and dGTP unperturbed. We have found (data not shown) that the decrease in incorporation into acid insoluble nuclear material in the wild type in the presence of cycloheximide is, indeed, more extensive when ^{3}[H]-thymidine is used than

when ^{3}[H]-deoxyadenosine is used. This, then, probably reflects both an inhibition of DNA synthesis and also an expanded dTTP pool causing dilution of the exogenously added ^{3}[H]-thymidine and possibly also a feedback inhibition of thymidine kinase. In the cycloheximide resistant strain there is essentially no effect of the drug on the incorporation of either ^{3}[H]-thymidine or ^{3}[H]-deoxyadenosine. This shows that a single factor mediating resistance through the ribosome (Haugli, Dove and Jimenez, 1972)[7] abolished both the pool expansion effect and the DNA synthesis effect of cycloheximide. Thus we chose to use ^{3}[H]-deoxyadenosine in the present work to minimize pool effects. A concentration of 5 µg cycloheximide per ml is sufficient to get maximal inhibition of incorporation of ^{3}[H]-deoxyadenosine in about 6 minutes.

2. Pulse experiments in the wild type.
The experimental strategy in these experiments is described in legends to fig. 1. From fig. 1 it is seen that cycloheximide pretreatment increasing from 0 to 2 and 4 minutes causes a severe depression in the relative amount of 10-35 S material accumulating in a 30 seconds pulse. Only relative amounts of molecular classes are shown in fig. 1. In fact, production of 4,5 S primary replication units is reduced to about 30-40 % of control level, after 4-6 minutes on cycloheximide.

3. Pulse short chase experiments in the wild type.
The effects of cycloheximide on the joining of 4,5 S fragments into intermediate 10-30 S molecules and finished 30-35 S "Replicon" size DNA molecules was studied in pulse-short chase experiments. The experimental regime is described in legends to fig. 2 and the results are shown in a normalized graphical presentation in fig. 2.

In a 2 min chase the control shows very little activity in the 4,5 S region and most of the labelled material is already present as 30-35 S material. The cycloheximide treated culture after a 2 minutes chase period shows deficiency of material in the 30-35 S region, and relative increase in low molecular weight material. After 10 minutes the deficiency of maturation in the cycloheximide treated culture is even larger when compared with

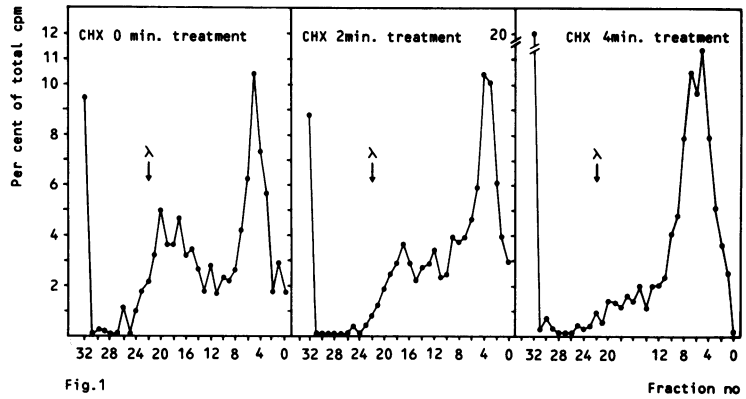

Figure 1: Alkaline sucrose gradient analysis of replication intermediates from wild type plasmodia pulselabelled after varying periods of cycloheximide (CHX) treatment. Position of ^{14}C-labelled λ-marker DNA (40 S) indicated by arrow. Discs of plasmodia were pulselabelled for 30 seconds at 30 minutes past metaphase II after treatment with cycloheximide at 5 µg/ml for 0,2 or 4 minutes.

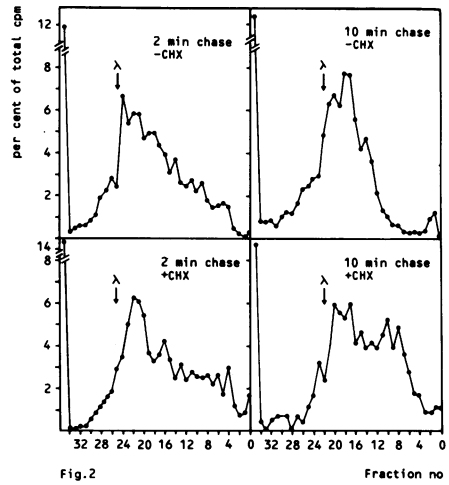

Figure 2: Alkaline sucrose gradient analysis of replication intermediates from wild type plasmodia exposed to pulse-chase labelling in presence and absence of cycloheximide (CHX). Pulse labelling for 30 seconds was done 30 minutes past metaphase II after 6 minutes pretreatment with cycloheximide. Cultures were then chased with cold deoxyadenosine for 2 or 10 minutes in continued presence of cycloheximide. Arrow indicates position of 40 S λ DNA.

untreated control. In the cycloheximide treated culture, little of the 4,5 S pieces remain as such. However, the ligation is slowed down and the normally transient intermediates from 10-25 S accumulate.

4. Pulse-long chase experiments in the wild type.
The experimental strategy is described in legend to fig. 3 and the results are expressed graphically in fig. 3. We conclude that the slow maturation or ligation of preformed 30-35 S pieces of DNA is also inhibited by cycloheximide since a 40 minutes chase yields a product of S value 24 - 40 compared to 40 - 56 in the control. The 70 minutes chase yields a 24 - 40 S product, while the control shows material in the 48 - 64 S region. Finally a 120 minutes chase leaves the material in the treated culture at 32 - 44 S, compared to 62 - 80 S in the control.

5. The effect of cycloheximide on DNA replication in a cycloheximide resistant strain.

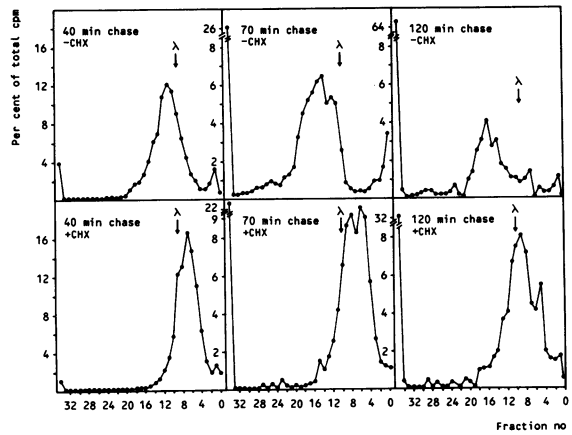

Figure 3: Alkaline sucrose gradient analysis of replication intermediates from wild type plasmodium after pulse-long chase treatment. The plasmodia were given a 30 seconds pulse at 30 minutes past metaphase II and then chased with cold deoxyadenosine for 40, 70 and 120 minutes in presence and absence of cycloheximide (CHX). In the cycloheximide treated cultures a 5 minute incubation without cycloheximide followed the pulse in order to allow ligation of most labelled DNA into 35 S pieces before the effects of cycloheximide were studied. Arrow shows position of λ.

The results reported above shows that cycloheximide has an effect on all three stages of DNA replication identified by us in an earlier report (Funderud and Haugli, 1975)[1]. These effects could all be caused by inhibition of protein synthesis brought about by the cycloheximide, or they could result from specific and direct effects of cycloheximide on the replication machinery. Direct evidence for an action mediated through protein synthesis was obtained by using a cycloheximide resistant strain where the resistance is known to reside with the ribosome and thus presumably exerts its action exclusively by allowing protein synthesis in the presence of cycloheximide. Fig. 4 presents results of experiments with the cycloheximide resistant plasmodium which is homozygous for the mutation actA 169.

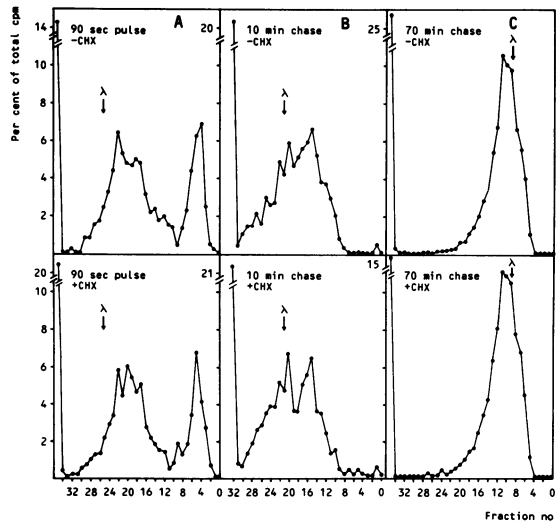

Figure 4: Alkaline sucrose gradient analysis of replication intermediates made in presence or absence of cycloheximide (CHX) in a cycloheximide resistant strain.

A. 90 seconds pulse given 30 minutes past metaphase II after 6 minutes pretreatment with cycloheximide.
B. 30 seconds pulse given 30 minutes past metaphase II after 6 minutes pretreatment with cycloheximide. Then chased with cold deoxyadenosine for 10 minutes in continued presence of cycloheximide.
C. 30 seconds pulse at 30 minutes past metaphase II, given after 6 minutes pretreatment with cycloheximide. Then chased with cold deoxyadenosine for 70 minutes in continued presence of cycloheximide. Arrow shows position of ^{14}C-labelled λ DNA (40 S).

Here, pulse labelling and pulse labelling followed by short and long chases in presence and absence of cycloheximide allows the conclusion that the cycloheximide resistant strain is completely insensitive to the drug, giving a pattern of replication intermediates which is identical with and without the drug - and which is almost identical to the pattern observed in the wild type.

DISCUSSION

The main conclusions emerging from the present work are: cycloheximide, in a cycloheximide-sensitive strain, (1) inhibits the first stage in DNA replication, formation of 4,5 S "Okazaki" pieces; (2) slows down the rapid joining of these into 30-35 S "Replicon" pieces of DNA and (3) prevents the slow maturation process where "Replicon" size DNA molecules are joined to high molecular weight DNA (see also Funderud and Haugli, 1975 (1)). Cycloheximide in a sensitive strain also causes more extensive inhibition of ^{3}[H]-thymidine than of ^{3}[H]-deoxyadenosine incorporation, reflecting the pool-effect observed by Bersier and Braun (1974)[16] and Evans et. al. (1976)[17].

Since a ribosomal cycloheximide resistant strain show none of these effects we conclude that all are brought about by prevention of synthesis of one or more proteins.

Evans et. al. (1976)[17] concluded that cycloheximide did not interfere with initiation of new replication units, but found that progeny strand elongation was inhibited. We have not addressed ourselves to the former question here, but our findings appears to be in agreement with the latter. With the present resolution of molecular species, however, we have been able to point to three sites of action. At the present time it can not be decided whether the three effects are inter-related or whether they are, in fact, independent.

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